APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1988, p. 1858–1860 Vol. 54, No. 7
0099-2240/88/071858-03$02.00/0

Oxidation of Thianthrene by the Ligninase of *Phanerochaete chrysosporium*

ROGER P. SCHREINER, S. EDWARD STEVENS, JR.,* AND MING TIEN

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 30 December 1987/Accepted 31 March 1988

The oxidation of heterocyclic sulfur compounds reported to be part of the macrostructure of coal and petroleum was investigated. The oxidation of thianthrene solubilized in 10% dimethylformamide to thianthrene monosulfoxide in the presence of hydrogen peroxide was catalyzed by the ligninase from *Phanerochaete chrysosporium*.

The lignin-degrading fungus *Phanerochaete chrysosporium* utilizes a highly nonspecific oxidizing system to degrade lignin to the level of carbon dioxide (3). *P. chrysosporium* produces an extracellular lignin-degrading enzyme, ligninase (5, 8), which has been shown to act on a variety of substrates (2). The aromatic lignin polymer is structurally similar to high volatile coals. In fact, it has been proposed that lignin was an essential precusor in the diagenesis of current coal and petroleum deposits (1) and that lignin from primordial conifers gave rise to current lignite deposits (4). For these reasons, it seemed possible that the ligninolytic system of *P. chrysosporium* might be used for the biological desulfurization of coal and petroleum.

The complexity of the molecular structure of coal and petroleum presents significant difficulties in conducting and interpreting enzymatic experiments. To simplify these difficulties, we chose to work with model sulfur heterocyclic compounds previously shown by various techniques to be present in petroleum and the coal polymeric matrix (6, 9, 12). The purposes of the present study were (i) to evaluate thianthrene as a potential substrate of ligninase, (ii) to identify the oxidation product(s) of ligninolytic activity, and (iii) to begin to define the mechanism of the ligninase-catalyzed oxidation of thianthrene.

Model compounds. Thianthrene (99% pure) was purchased from Aldrich Chemical Co. Ligninase was produced and purified as described previously by Tien and Kirk (10), with *P. chrysosporium* BKM-F-1767. All other chemicals used were of analytical grade.

Enzyme assays. Assays with thianthrene (0.46 μM) were carried out in 0.10 M sodium tartrate (pH 3.5) with 10% (vol/vol) dimethylformamide (DMF) and 1.0 nM ligninase (ε_{409} = 168 mM^{-1} [11]). The reactions were initiated by the addition of 0.32 μM H_2O_2 and monitored by scanning from 400 to 245 nm on a Gilford model 250 spectrophotometer.

Purification of the product of thianthrene oxidation by ligninase. With thianthrene as the substrate, the assay was upscaled to 200 ml total volume. Thianthrene was successively added in 0.46 μM concentrations until the 280-nm product peak no longer increased (12× substrate added). The product and residual thianthrene were extracted from the assay mixture three times with anhydrous ethyl ether. The ether extract was rotoevaporated, and the remaining white crystals were suspended in 3 ml of methanol. The methanol was injected into a reverse-phase C18 high-pressure liquid chromatography (HPLC) column with 75% methanol–25% water as the solvent. The product was detected by absorbance at 284 nm, collected from the HPLC column, and rotoevaporated. The remaining crystals were suspended in 1 ml of methanol, injected, and collected from the HPLC column again by using a 60% methanol–40% water solvent (retention time, 7.75 min). The appropriate eluate fraction was again rotoevaporated, and the product crystals were recrystallized from ethanol.

Preparation of thianthrene monosulfoxide. Thianthrene monosulfoxide was prepared, as described by Hilditch (7), by oxidation of thianthrene in acetic and dilute nitric acids, followed by recrystallization from ethanol (mp, 143°C). The melting point of chemically prepared and enzymatically prepared compounds was measured with a Fisher-Johns apparatus. Both had an mp of 143°C.

Analysis of thianthrene monosulfoxide and purified product. HPLC was performed on a reverse-phase C18 column equipped with a Waters WISP 710B on-line injector, SSI model 200 pumps and SSI model 210 guardian, and Gilson holochrome and model 111B UV detectors. The solvent used was 75% methanol–25% water, and the detectors were set to monitor 255 and 284 nm.

The UV spectra were obtained with a Cary 14 dual-beam spectrophotometer equipped with a Northstar model 3820 data system. The reaction product and thianthrene monosulfoxide crystals were dissolved in methanol, and spectra were taken from 400 to 220 nm.

Proton nuclear magnetic resonance (NMR) was performed in deuterated chloroform from 0 to 12 ppm. Spectra were taken by A. J. Freyer (Department of Chemistry, Pennsylvania State University) on a Bruker WP200 NMR spectrophotometer.

Gas chromatography (GC) was performed on a Sigma 3B gas chromatograph equipped with flame ionization detectors. The column (8 ft, 1.8 mm innerdiameter, glass) was packed with 20% SP2100 on 80/100 Supelcoport (Supelco). Nitrogen was used as the carrier gas at 35 ml/min. The oven temperature was 240°C, with the injector at 265°C and the detector at 290°C.

The GC-mass spectra were generated by a Kratos MS9/50 instrument equipped with a Data General Nova/4 mainframe. Ionization of samples was done by electron impact with a source temperature of 240°C.

Thianthrene was insoluble in water; thus, a solvent compatible with enzymatically active ligninase was necessary. DMF (10% by volume in H_2O) was eventually chosen because it had no discernible effect on ligninase activity (as shown by a lack of effect on the rate of veratryl alcohol

* Corresponding author.

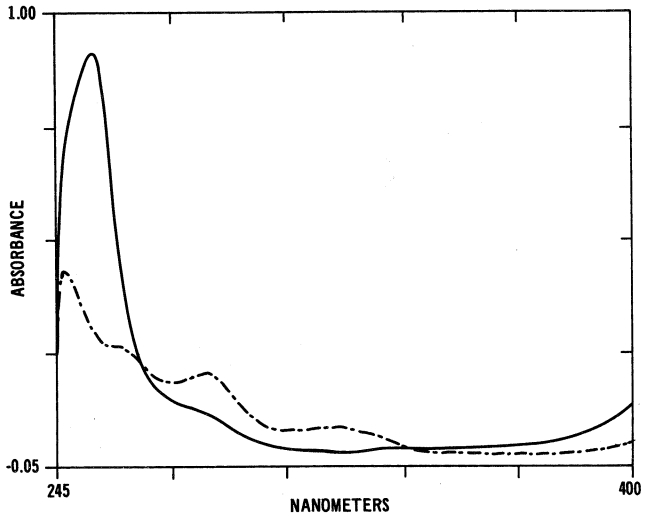

FIG. 1. Induced absorbance changes resulting from the ligninase-catalyzed conversion of thianthrene. Spectra of reaction mixtures were taken before (——) and 1 min after (–·–) the addition of 0.32 μM H_2O_2.

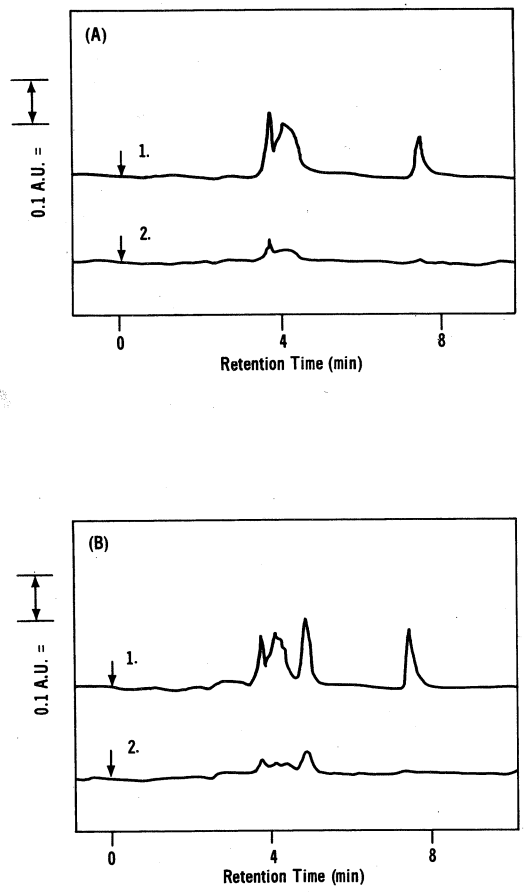

FIG. 2. HPLC elution profile of a typical thianthrene reaction mixture before (A) and after (B) the addition of 0.32 μM H_2O_2. Thianthrene reaction mixture (100 μl) with or without H_2O_2 was injected into the HPLC column at the time indicated by the arrows. The column eluate was monitored at 255 nm (1.) and at 280 nm (2.). A.U., Absorbance unit.

oxidation by ligninase), thianthrene was soluble in it (empirically determined), and it was miscible with water. DMF (10% in H_2O) was used as the solvent in all subsequent experiments in which ligninase was used.

In 10% DMF, thianthrene was strongly absorbing at 255 nm. This absorption peak was bleached with a concomitant increase in a 284-nm absorption peak upon initiation of ligninase catalysis by the addition of H_2O_2 to the reaction mixture (Fig. 1). No spectral changes were observed in the absence of enzyme or H_2O_2.

Product formation from thianthrene was confirmed by HPLC analysis (Fig. 2). HPLC analysis of a typical reaction mixture minus H_2O_2 is shown in Fig. 2A. Addition of H_2O_2 to the reaction mixture resulted in the formation of a new peak, resolvable by HPLC (Fig. 2B). The retention time of the product was 4.9 min and that of thianthrene was 7.5 min. Therefore, the product was more water soluble.

After purifying the product and interpreting NMR and mass spectral data, we deduced that it was most probably thianthrene monosulfoxide. We prepared thianthrene monosulfoxide as described above and compared its UV absorption spectrum and its retention time in GC with that of the purified product. Both samples gave maximal UV absorbance at 238 to 239 nm, with a broad shoulder at 284 to 285 nm and retention times of 13 min and 36 s, respectively, on GC (data not shown). Mass spectra indicated identical *m/e*'s of 232 for the molecular ion of chemically prepared and enzymatically generated material, with maximum ion peaks of 184 for both. The H NMR spectra (Fig. 3) of chemically prepared and enzymatically generated material were also identical. Therefore, the reaction of thianthrene with ligninase in the presence of H_2O_2 yields thianthrene monosulfoxide (Fig. 4).

Attempts at trapping a thianthrene cation radical generated by ligninase have been unsuccessful. However, we believe that the monosulfoxide generated by ligninase pro-

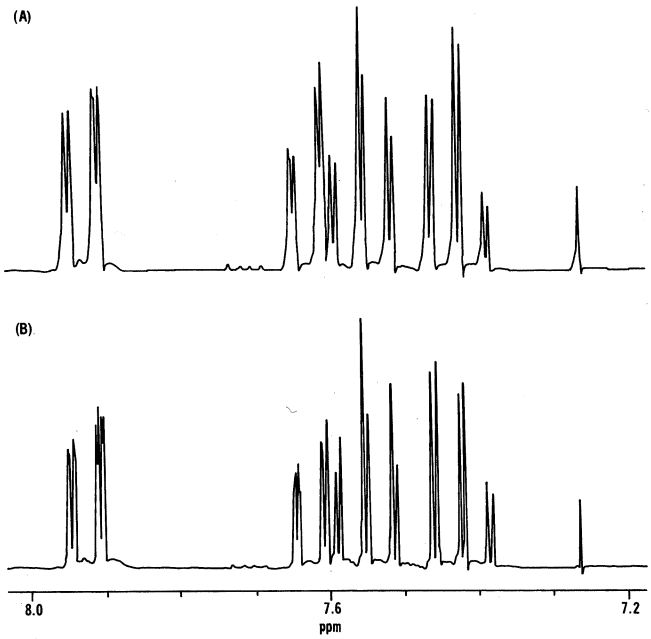

FIG. 3. Proton NMR spectra of thianthrene monosulfoxide, showing the aromatic region (6 to 8 ppm). Thianthrene monosulfoxide was prepared as indicated in the text. The proton NMR spectra of authentic material (A) and of purified product (B) from the ligninase-catalyzed conversion of thianthrene are shown.

FIG. 4. Chemical structures of thianthrene and thianthrene monosulfoxide.

ceeds through a cation radical, since ligninase generates cation radicals with other substrates (5, 8) and the monosulfoxide prepared chemically proceeds through a cation radical intermediate (7).

The fact that ligninase oxidizes thianthrene to thianthrene monosulfoxide is not surprising. The nonspecific action of ligninase is well documented. However, oxidation of thianthrene to a more water-soluble product by the enzyme ligninase provides a rationale for investigating the use of *P. chrysosporium* and the ligninolytic system from this organism in the biological desulfurization of coal and petroleum. Biological desulfurization efforts are aimed at removing the organic sulfur found in coal and oil. Probably the best method of accomplishing this goal is to convert the sulfur into a water-soluble form that can be easily washed away. The conversion of thianthrene to a more water-soluble product is a step in the right direction. Other lignin-degrading enzymes are produced by *P. chrysosporium*, and the further oxidation of thianthrene monosulfoxide may be feasible. The cooperation of a number of enzymes in the lignin-degrading system may prove to be an effective method of removing organic sulfur from coal and petroleum, a plausible notion because the representative structures of coal and lignin are closely related.

This research was supported by grants 85-CRCR-1-1760 (S.E.S.) and 85-FSTY-9-0103 (M.T.) from the USDA Competitive Grants Program.

LITERATURE CITED

1. **Alexanian, C.** 1958. Contribution a nos connaissances sur le mode de formation de la Houille. Constatations faites par l'absorption infrarouge. C. R. Hebd. Seances Acad. Sci. Ser. B **246:**1192–1195.
2. **Bumpus, J. A., M. Tien, D. Wright, and S. A. Aust.** 1985. Oxidation of persistent environmental pollutants by a white rot fungus. Science **228:**1434–1436.
3. **Crawford, R. L.** 1981. Lignin biodegradation and transformation. John Wiley & Sons, New York.
4. **Gillet, A.** 1955. Die Entwicklung der Humine aus verhotzter Materie Von der Zellulose zum Anthrazit. Brennstoff-Chemie **36:**103–120.
5. **Hammel, K. E., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. Mechanism of oxidative C_α-C_β cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **260:**8348–8353.
6. **Hayatsu, R., R. G. Scott, L. P. Moore, and M. H. Studier.** 1975. Aromatic units in coal. Nature (London) **257:**378–380.
7. **Hilditch, T. P.** 1910. The intermolecular condensation of aromatic sulphinic acids. J. Chem. Soc. London **97:**2579–2594.
8. **Kersten, P. J., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. J. Biol. Chem. **260:**2609–2612.
9. **Spiro, C. L., J. Wong, F. W. Lytle, R. B. Greegor, D. H. Maylotte, and S. H. Lamson.** 1984. X-ray absorption spectroscopic investigation of sulfur sites in coal: organic sulfur identification. Science **226:**48–50.
10. **Tien, M., and T. K. Kirk.** 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: purification, characterization, and catalytic properties of a unique H_2O_2-requiring oxygenase. Proc. Natl. Acad. Sci. USA **81:**2280–2284.
11. **Tien, M., T. K. Kirk, C. Bull, and J. A. Fee.** 1986. Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**1687–1693.
12. **Willey, C., M. Iwao, R. N. Castle, and M. L. Lee.** 1981. Determination of sulfur heterocycles in coal liquids and shale oils. Anal. Chem. **53:**400–407.